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VACCINE DELIVERY

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a continuation-in-part of U.S. Patent Application No. 09/888,235 entitled "DELIVERY VEHICLE COMPOSITION AND METHODS FOR DELIVERING ANTIGENS AND OTHER DRUGS" filed June 22, 200, which U.S. Patent Application Serial No. 09/888,235 is a continuation-in-part of U.S. Patent Application Serial No. 09/602,654 entitled "IMMUNOGEN COMPOSITION AND METHODS FOR USING THE SAME" filed June 22, 2000 and also claims priority from U.S. Provisional Patent Application Serial No. 60/278,267 entitled "IMMUNOGEN COMPOSITION AND METHODS FOR DELIVERY OF ANTIGEN TO ELICIT MUCOSAL IMMUNE RESPONSE" filed March 23, 2001, and the entire contents of each and all of these referenced Patent Applications are incorporated by reference herein as if set forth herein in full. Moreover, the subject matter disclosed in each of these referenced Patent Applications is useful in combination with the subject matter disclosed herein, and all combinations of any feature or features disclosed in any of these referenced Patent Applications with any feature or features disclosed herein are within the scope of the present invention.

FIELD OF THE INVENTION

The invention relates to vaccine delivery, including vaccine delivery vehicle compositions, manufacture of such delivery vehicle compositions and treatments involving such delivery vehicle compositions.

ABSTRACT

We have developed a vaccine delivery system based on the non-ionic block copolymer, Pluronic® F127 (F127), combined with selected immunomodulators. F127-based matrices are characterized by a phenomenon known as reverse thermogelation, whereby the formulation undergoes a phase transition from liquid to gel upon reaching physiological temperatures. Protein antigens (tetanus toxoid (TT), diphtheria toxoid (DT) and anthrax recombinant protective antigen (rPA) were formulated with F127 in combination with CpG motifs or chitosan, as examples of immunomodulators, and were compared to more traditional adjuvants in mice.

IgG antibody responses were significantly enhanced by the F127/CpG and F127/chitosan combinations compared to antigens mixed with CpGs or chitosan alone. In addition, the responses were significantly greater than those elicited by aluminum salts. Furthermore, the functional activity of these antibodies was demonstrated using either *in vivo* tetanus toxin challenge or an anthrax lethal toxin neutralization assay. These studies suggest that a block-copolymer approach could enhance the delivery of a variety of clinically useful antigens in vaccination schemes.

1. INTRODUCTION

Although significant progress in vaccine development and administration has been made, alternative approaches that enhance the efficacy and safety of vaccine preparations remain under investigation. Sub-unit vaccines such as recombinant proteins and synthetic peptides are emerging as novel vaccine candidates. However, traditional vaccines, consisting of attenuated pathogens and whole inactivated organisms, contain impurities and bacterial components capable of acting as adjuvants, an activity which these subunit vaccines lack. Therefore the efficacy of highly purified sub-unit vaccines will require addition of potent adjuvants.

Currently, aluminum compounds are the only adjuvants approved for use in human vaccines in the United States [1]. Despite their good safety record, they are relatively weak adjuvants [1] and often require multiple dose regimens to elicit antibody levels associated with protective immunity. Aluminum compounds may therefore not be ideal adjuvants for the induction of protective immune responses to sub-unit vaccines. Although many candidate adjuvants are presently under investigation, they suffer from a number of disadvantages including toxicity in humans and requirements for sophisticated techniques to incorporate antigens.

We have recently reported the immunostimulatory effects on the mucosal immune response of a unique adjuvant system composed of the block co-polymer, Pluronic®F127 (F127), and the cationic polysaccharide, chitosan [2]. F127 is a non-ionic, hydrophilic polyoxyethylene-polypropylene (POE-POP) block copolymer previously used for its surfactant and protein stabilizing properties [3-5]. F127-based matrices are characterized

by a phenomenon known as reverse thermo-gelation whereby they undergo a phase transition from liquid to gel upon reaching physiological temperatures. Therefore formulations of F127 can be administered in liquid form at temperatures less than approximately 10°C, with conversion to semi-solid gels at body temperature, thereby potentially acting as sustained release depots. Furthermore, proteins contained within the pluronic matrix at high concentrations have been shown to retain their native configuration [3].

Chitosan has previously been shown to have both mucosal and systemic adjuvant activity [6-9]. We used a F127/chitosan combination as a delivery vehicle for mucosal vaccine administration and demonstrated that both components contributed to the immunoenhancing effect observed [2]. In the present studies, we demonstrate the utility of the F127/chitosan system as a vaccine delivery vehicle for protein antigens for systemic immunization. In addition, in order to evaluate the potential of F127 to enhance the activity of other adjuvants, we incorporated immunostimulatory DNA preparations containing CpG motifs (CpGs) [10-13] into the formulations and show here that the activity of this adjuvant was dramatically enhanced within the pluronic matrix. Furthermore, these formulations elicit protective antibody responses. Although both chitosan and CpGs are known to have potent adjuvant activity, the combination of either with F127 is unique and results in improved immune responses compared to either adjuvant used alone. To prepare formulations, vaccine antigens and immunomodulators are simply mixed with the vehicle. This straightforward approach may therefore enhance delivery of a variety of clinically useful antigens in vaccination schemes.

2. MATERIALS AND METHODS

2.1 Antigens

Tetanus toxoid (TT), was obtained from Accurate Chemical & Scientific (Westbury, NY), and contained 961 Lf/ml and 1884 Lf/mg protein nitrogen. Diphtheria toxoid (DT; Accurate) contained 2100 Lf/ml and 1667 Lf/mg protein nitrogen. Recombinant anthrax protective antigen (rPA) was obtained from Dr. Stephen Leppla (NIH), under a license agreement with the NIH, as a lyophilized protein in 5 mM Hepes, pH 7.4. It was reconstituted in water (USP grade; Abbott Laboratories, Chicago, IL) at 2 mg/ml before use.

2.2 Preparation of formulations

Pluronic® F127 (BASF, Washington, NJ) stock solution was prepared at 30 or 34% (w/w) in ice-cold PBS with complete dissolution achieved by storing overnight (ON) at 4°C. Chitosan (medium molecular weight chitosan; Sigma-Aldrich, St. Louis, MO) or Protasan® (Chitosan chloride, UP CL 213; ProNova Biomedical, Oslo, Norway) stock solution was prepared at 3% (w/w) in 1 % (v/v) acetic acid in 0.9% (w/v) saline and heated at 37°C to dissolve. These sources of chitosan had equivalent activity in our formulations. Proprietary preparations of oligodeoxynucleotides containing CpG dinucleotide motifs (CpGs) were obtained from Qiagen (ImmunEasy™; Qiagen Inc., Valencia, CA) and were added to formulations or mixed with antigens alone according to the manufacturer's instructions. This proprietary preparation of CpG additionally contains aluminum hydroxide. Unless otherwise noted, the stock solutions were mixed together to prepare formulations

containing various combinations of antigen, 0.5% (w/w) chitosan, 20% (v/v) CpGs and 16.25% (w/w) F127.

TT adsorbed to aluminum phosphate (AP; Wyeth Laboratories Inc., Marietta, PA) was obtained as a preparation containing 10 Lf/ml. rPA/alum was prepared by adsorption of rPA to Imject® alum (Pierce Endogen, Rockford, IL) by standard methods.

To prepare emulsions with Incomplete Freund's Adjuvant (IFA; Sigma-Aldrich), antigens with or without immunomodulators were diluted in PBS and emulsified with IFA at a 1:1 (v/v) ratio.

2.3 Immunization studies in mice

Balb/c female mice (Taconic Farms Inc., Germantown, N.Y. or Harlan SD, Indianapolis, IN) and ICR (CD-1®) outbred female mice (Harlan), 6 to 8 weeks of age, were used for these studies. Mice were immunized once intra-peritoneally (i.p.) or subcutaneously (s.c) with various formulations as described above.

2.4 ELISA

The serum antibody responses to TT, DT and rPA were measured by ELISA as previously described [2]. Briefly, serum samples were obtained by bleeding from the retro-orbital plexus under inhalation anesthesia and were stored at -20°C until assay. Wells of 96 well Nunc Maxisorb microtiter plates (Nunc, Gaithersburg, MD) were coated with either 1 μg/ml TT or rPA or 10μg/ml DT in PBS. Plates were washed with PBS/0.05% Tween 20 and blocked with 1% bovine serum albumin (BSA; Fisher Scientific, Pittsburgh, PA). Samples were serially diluted in PBST (PBS/0.1% BSA/0.05% Tween 20) and added to wells in triplicate. Following incubation, plates were washed and goat anti-mouse IgG-gamma chain specific horseradish peroxidase

(HRP)-labeled conjugate (Sigma-Aldrich) was added in PBST. After further incubation, antibody binding was detected with substrate buffer containing TMB (3,3',5,5'-tetramethylbenzidine; Sigma-Aldrich). After the reaction was stopped with 0.5 M H_2SO_4 (Sigma-Aldrich), absorbance was read at 450 nm with an EIA reader (Molecular Devices, Sunnyvale, CA). Assays to measure antibody IgG subclasses were performed as described above using IgG1 and IgG2a specific HRP-labeled conjugates (Southern Biotechnology Associates, Birmingham, AL). Antibody titer was defined as the reciprocal of the dilution of serum that would yield an optical density of 0.5.

Analysis of differences in titers between groups was performed using the Mann-Whitney Rank Sum Test. A probability (p) of 0.05 or less was accepted as significant.

2.5 ELISPOT assay for anti-TT antibody-secreting cells (ASC)

Numbers of TT-specific ASC were assessed by ELISPOT assay. Wells of flat-bottomed microtiter plates were coated as described above, blocked with 0.1% BSA/PBS and then washed with PBS before addition of cells. Single cell suspensions from bone marrow and spleen were prepared in Hank's balanced salts solution (BSS; Invitrogen, Carlsbad, CA). Bone marrow was obtained from the femurs of immunized or control mice according to the method of Mishell and Shigii [14] and erythrocytes removed with lysing buffer. Cells were washed and resuspended in 5% fetal bovine serum (FBS; Hyclone, Ogden, UT) in RPMI (Invitrogen) at 5 x 10^6 cells/ml. For enumeration of IgG anti-TT ASC, goat anti-mouse IgG (gamma-chain specific) antibody (Kirkegaard and Perry Laboratories (KPL), Gaithersburg, MD) was added to the cell suspensions at a final dilution of 1:500. Cells were plated at 1.25, 2.5 and 5 x 10^5 cells/well in triplicate and plates incubated in a humidified incubator with 5% CO_2 for 3 hr at 37^0C. After incubation, plates were washed

with 0.01% Tween/PBS and phosphatase-labeled rabbit anti-goat IgG antibody (KPL) was added. Plates were incubated ON at RT and washed before addition of the substrate, BCIP (5-bromo 4-chloro 3-indolyl phosphate; Sigma-Aldrich), dissolved at 1 mg/ml in AMP (2-amino-2-methyl-1-propanol; Sigma-Aldrich) buffer, pH 10.25, 0.01% Triton X-100. Plates were developed at RT for 1-2 h and rinsed with distilled water. Spots were counted with the aid of a dissecting microscope at 50x magnification. Results are expressed for individual animals as mean ASC/10^6 cells.

2.6 Anthrax Toxin Neutralization assay (TNA)

Serum samples from animals immunized with rPA were tested for their ability to prevent the lethal toxin (PA + lethal factor (LF))-induced mortality of J774A.1 cells (American Type Culture Collection, Manassas, VA) [15]. LF was obtained from NIH under an MTA. Aliquots of 100 µl cell suspension (6 to 8 x10^5 cells/ml) in Dulbecco's modified Eagle's medium with 10% FBS (Invitrogen) were plated into flat 96-well cell culture plates (Corning Costar, Acton, MA). Serial dilutions of pre- and post-immune serum samples were made in TSTA buffer (50 mM Tris pH 7.6, 142 mM sodium chloride, 0.05% sodium azide, 0.05% Tween 20, 2% BSA). PA and LF at final concentrations of 50 and 40 ng/ml respectively were added to each antiserum dilution. After incubation for 1h, 10 µl of each of the antiserum-toxin complex mixtures were added to 100 µl J774A.1 cell suspension. The plates were incubated for 5h at 37°C in 5% CO_2. Twenty-five µl of MTT (3-[4,5-dimethyl-thiazol-2-y-]-2,5-diphenyltetrazolium bromide; Sigma-Aldrich) at 5 mg/ml in PBS was then added per well. After 2h incubation, cells were lysed and the reduced purple formazan solubilized by adding 20% (w/v) SDS in 50% dimethylformamide, pH 4.7 [16]. ODs were read at 570 nm on an

EIA reader. The lethal toxin-neutralizing antibody titers of individual serum samples, calculated by linear regression analysis, were expressed as the reciprocal of the antibody dilution preventing 50% cell death and these titers were normalized to a control rabbit anti-PA antiserum (from NIH).

Pre and post-immunization serum toxin neutralization titers were compared by the Sign test. Toxin neutralization titers between groups were compared by the use of the Mann Whitney U test. P values less than or equal to 0.05 was considered to indicate a significant difference.

2.7 Tetanus toxin challenge

Lethal challenge with tetanus toxin was performed as described by Anderson et al. [17]. Briefly mice were immunized i.p. on day 0 with 0.5 Lf TT in either PBS or F127/chitosan. Negative controls consisted of mice immunized i.p. with vehicle (F127/chitosan) alone. At 6 weeks, all mice were challenged i.p. with 100 x LD_{50} tetanus toxin (List Biological Labs. Inc., Campbell, CA). Mice were monitored for 1 week thereafter and deaths recorded.

3. RESULTS

3.1 Duration of the antibody response following s.c. immunization with TT/AP and TT/F127/chitosan

Groups of outbred ICR mice were immunized once s.c. with 1.5 Lf TT formulated either in F127/chitosan or adsorbed to AP. Animals were bled at various times and the IgG anti-TT antibody response was monitored over a ten month period. This dose of TT had previously been selected as optimal in these studies (data not shown). The results of this study (Figure 1) indicate that TT/F127/chitosan raised a rapid and potent IgG antibody

response with antibodies being easily detected at one week. These titers rose to a peak approximately 8 to 12 weeks after injection and were then sustained for at least ten months with titers of approximately 100,000. In contrast, the response to TT/AP was slower to appear and did not attain the levels of TT/F127/chitosan immunized mice. At the peak of the response the titers in AP-immunized mice were only one-third of those of TT/F127/chitosan immunized mice ($p < 0.05$ for all time points).

3.2 The long-lived antibody response to TT/F127/chitosan is maintained by antibody-secreting cells resident in the bone marrow

The durable nature of the antibody response to a single injection of TT/F127/chitosan could be explained either by the persistence of antigen or by long-lived antibody secreting cells (ASC), which reside in the bone marrow [18,19]. We therefore enumerated ASC in the bone marrow and spleens of Balb/c mice that had been immunized one year previously with 1.5 LF TT in F127/chitosan or PBS. The data indicate (Table 1) that ASC were present in the bone marrow one year after immunization with TT/F127/chitosan whereas none could be detected in the spleens of these mice. In contrast, no ASC were found in either the bone marrow or spleen of mice immunized with TT/PBS. However, early in the response, at one and two weeks post-immunization, ASC were abundant in the spleen and draining lymph nodes but not bone marrow of mice immunized with TT/F127/chitosan (data not shown). By day 28 a distribution of the ASC from the spleen to the bone marrow could be observed (data not shown). Animals receiving vehicle (F127/chitosan) alone had no ASC in bone marrow or spleen at any time points (data not shown).

3.3 Single dose of TT/F127/chitosan is more potent than multiple injections of TT/AP

In order to compare our formulation with a standard vaccination regimen, Balb/c mice were immunized s.c. either with a single dose of 1.5 Lf TT/F127/chitosan or with three doses of 1.5 Lf TT/AP given at monthly intervals (total of 4.5 Lf TT given). It is apparent from the data shown in Figure 2 that the response to TT/AP did not achieve the IgG anti-TT levels of those elicited by TT/F127/chitosan until at least two injections had been administered ($p = 0.008$ at week 2; $p = 0.012$ at week 4; $p > 0.05$ at week 8).

3.4 TT/F127/chitosan elicits a protective immune response

To examine whether these formulations generated a protective immune response, mice were subjected to a lethal challenge with tetanus toxin, performed as described in Anderson et al [17]. Balb/c mice were immunized i.p. with 0.5 LF TT in either PBS or F127/chitosan. In addition, a group of animals received F127/chitosan vehicle alone. At six weeks mice were challenged i.p. with 100 x LD_{50} of tetanus toxin. The results of these studies (Figure 3) indicate that immunization with TT/F127/chitosan resulted in protective immunity as all mice (8/8) survived. These results were significantly different ($p=0.005$) from the TT/PBS treated mice, which did not survive the lethal toxin challenge (0/8). As expected, animals immunized with vehicle alone also succumbed to the toxin challenge (0/8 survived).

3.5 TT/F127/chitosan is superior to either component of the formulation alone

We next compared TT/F127/chitosan to the same dose of TT given with each component of the formulation mixed with TT alone. In this study, groups of Balb/c mice were given a single s.c. injection of 0.5 Lf TT/F127/chitosan, TT/chitosan or TT/F127. Responses were monitored over a three month period following injection (Figure 4). TT in the dual-component formulation was found to elicit a significantly more potent antibody response

than TT/chitosan at 5 weeks after immunization at which time the response to TT/F127/chitosan was approximately 3 times higher than that to TT/chitosan alone ($p = 0.0206$). By week 8, the TT/F127/chitosan response was still twice as high as that to TT/chitosan but this was no longer statistically significant. These responses were plateaued at week 8 as they did not increase further by week 12. Also at all times, the responses to TT in both chitosan-containing formulations were significantly greater than that to TT/F127 alone.

3.6 Formulation of CpGs with F127 and antigen

In order to establish whether combinations of F127 with other adjuvants could elicit enhanced responses, groups of Balb/c mice were immunized once s.c. with 0.5 Lf TT either mixed with CpGs or formulated with F127/CpG. In addition, a group of mice was immunized with TT/CpG emulsified in IFA to compare F127 to a classical depot-type adjuvant. Suboptimal doses of the antigens were used in these comparisons to better distinguish between the preparations. Data from a representative experiment (Figure 5a) indicate that at 4 and 8 weeks, the presence of the pluronic component significantly enhanced the IgG antibody response to TT compared to CpG/antigen alone ($p = 0.0023$ and 0.029 respectively). Furthermore, the response to TT/F127/CpG was significantly higher than that elicited by TT/CpG/IFA ($p = 0.017$ and 0.029 at 4 and 8 weeks respectively).

Similar enhancement was seen when DT was used as the antigen (Figure 5b). At 4 weeks after a single injection, formulation of DT with F127/CpG elicited a significantly enhanced IgG antibody response compared to that elicited by DT/CpG alone ($p < 0.05$).

When the dose of CpGs was reduced in the formulations it was found that, even with a log reduction in the amount of CpG, a better response was still achieved in the presence of F127 (Figure 5c).

3.7 Formulation of anthrax rPA in F127 pluronic

In a preliminary study, we compared the antibody response to a single dose of 25 μg rPA formulated with either F127/chitosan or F127/CpG or adsorbed to alum. In addition a group received the antigen in F127 alone. All animals were boosted 8 months later and the functional nature of the antibody response to rPA was measured by TNA. Figure 6a shows data from serum samples taken week 8 after the primary injection and demonstrates that formulation of rPA with F127/CpG induced toxin neutralizing titers that were significantly higher than the mix of rPA/CpG alone ($p = 0.041$) as well as rPA/alum ($p = 0.002$), rPA/F127/chitosan ($p = 0.001$) and rPA/F127 ($p = 0.002$).

At a later time point from same study when samples were taken 2 weeks after the boost (Figure 6b), all TNA values increased substantially as would be expected. The responses to rPA/F127/CpG and rPA/CpG alone were still much higher than all other groups although at this point, there was no significant difference between rPA/F127/CpG and rPA/CpG alone. However, these studies were carried out with a single high dose of rPA (25 μg) and it is likely that this difference could be expanded by the use of limiting doses of antigen and/or adjuvant as illustrated in Figure 5 with TT as antigen.

Interestingly, after the boost, rPA/F127 alone could elicit considerable levels of neutralizing antibodies against rPA. Values of approximately 300 were generated, which were similar to those elicited by alum in this study and were higher than the values

elicited by the F127/chitosan formulation although these values were not significantly different from each other.

3.8 IgG subclass analysis

IgG subclass analysis was performed on week 8 sera from mice immunized s.c. with rPA in various formulations. The data indicate that rPA/F127/chitosan and rPA/alum elicited mainly Th2-type responses with IgG1 being the predominant subclass (Figure 7). In animals receiving rPA/F127/CpG, the response was dominated by IgG2a indicating that a Th1-type response was elicited as has been previously reported in the literature [10-12]. IgG subclass analysis was also performed on samples from mice immunized s.c. with TT/F127/CpG combinations. These data (Figure 7) also indicate that CpGs strongly influenced the IgG antibody response, with a significant IgG2a anti-TT response. IgG1 was still easily detectable in all samples, however.

4. DISCUSSION

In a previous study we demonstrated that a novel vaccine delivery system consisting of a sustained release component, Pluronic® F127, combined with a penetration enhancing adjuvant, chitosan, and the antigen, TT, significantly increased the antibody response to intranasally delivered antigen [2]. In this report we establish that this formulation also significantly enhances the antibody response to systemically administered antigens. Furthermore we show that the immunostimulatory activity of another potent adjuvant, CpG, was also significantly enhanced upon formulation in the pluronic matrix.

A single immunization with antigen in F127/chitosan induced an antibody response significantly greater than the immune response to TT/alum in both inbred and outbred

mice. Moreover, at least two immunizations with TT/alum were required to induce an anti-TT antibody response comparable to that obtained after a single dose of TT in F127/chitosan. In addition, at early time points, the response to TT/F127/chitosan was significantly higher than that to TT mixed with chitosan in the absence of the pluronic.

The duration of the antibody response following a single dose of TT in F127/chitosan, was evaluated over a ten month period and showed minimal decay in antibody levels over time. These results indicate a continual production of anti-TT antibodies as the half-life of IgG is only approximately 23 days [20]. We found that this response was maintained by long-lived antibody-secreting cells, resident in the bone marrow. The generation of these long-lived cells greatly diminishes the degree of regeneration required to maintain persistent antibody levels [21] and thus these cells represent an important first line of defense against re-infection before the memory B cell population is activated to effector stage.

Formulations of TT with F127/chitosan elicited protective immunity as mice immunized with TT/F127/chitosan survived an otherwise lethal challenge with tetanus toxin six weeks after a single injection, indicating that the antigen was maintained in its native conformational state within the formulation. Taken together with results showing longevity of the immune response after a single immunization, the results suggest that these formulations are capable of eliciting durable, protective antibody responses. Although protection was not monitored at later time points, the lack of diminishment in the antibody levels suggests that protection would be maintained over a long period of time.

The presence of F127 enhanced the immunogenicity of TT administered with chitosan and afforded an early advantage in induction of the IgG antibody response. This enhancement although modest (approximately three-fold) compared to chitosan alone (see Figure 4), may be due to the ability of F127 to stabilize the protein antigen. We have not investigated if conformation of the protein antigen is maintained in mixtures with chitosan without F127 although McNeela et al. [8] and Seferian and Martinez [9] have reported that combinations of antigen and chitosan can elicit functional antibodies. The improvement of the antibody response at early time points by chitosan in the presence of F127 has previously been seen in intranasal administration of this formulation [2]. However, chitosan was an ineffective adjuvant when used in combination with anthrax rPA (see Figure 6a and b). This was probably due to the low resultant pH of this formulation since rPA is a pH sensitive antigen and will unfold at pH less than 6.

The enhanced adjuvant effects of chitosan administered in combination with TT/F127 suggested that F127 might be synergistic with other immunomodulating agents. We therefore also studied the immunogenicity of CpG preparations in combination with TT and F127. The ability of these oligonucleotides to enhance both mucosal and systemic immune responses to a wide variety of antigens is well documented in the literature [10,22-27]. A recent study in mice [22] showed that the combination of other adjuvants with CpGs significantly enhanced the immune response to hepatitis B virus surface antigen (HBsAg). Several adjuvants were tested in combination with CpGs, including alum, IFA, CFA and MPL. The combination of IFA with CpGs resulted in the highest IgG anti-HBsAg antibody response and this response was higher than either component

alone. However, the combination of CpGs and alum also induced a synergistic IgG antibody response of similar magnitude to the CpG/IFA combination. In a separate study, using a bovine herpes virus glycoprotein in cattle, combination of CpGs with another oil-in-water based adjuvant, Emulsigen, enhanced the response to antigen compared to CpGs used alone [28]. Combinations of adjuvants with different modes of action can therefore clearly be beneficial in terms of raising optimal immune responses, a point that was recently emphasized (see other papers in this volume). We therefore compared CpGs in combination with F127 and, since the commercial preparation of CpG used here additionally contains alum, we were able to measure the additional effects of F127 delivery on this potent combination. We now show here that the immune responses to TT and DT were significantly increased up to ten-fold (see Figure 5a and b) when the antigen was formulated with F127/CpG/alum as compared to antigen/CpG/alum alone. Furthermore the dose of the CpG/alum could also be lowered in the presence of F127 (Figure 5c). A tenfold reduction of the CpG dose in the presence of F127 induced a higher antibody response to TT than the standard dose of CpG without the F127 matrix. This suggests that other immunomodulators could also be used at reduced doses in the F127 matrix thereby potentially leading to lower reactogenicity and other side effects.

The mechanism by which F127 augments the activity of antigens and adjuvants contained within its matrix has not been elucidated. The enhanced antibody response may be a consequence of sustained delivery, targeted delivery, improved stability of the protein or immunomodulator contained in the matrix or a combination of all these effects. The ability of F127 to redirect particles to the reticuloendothelial system in general and bone marrow in particular has previously been shown in rabbits [29], a finding that would tend

to suggest that targeted delivery has a role to play in the current studies. Some aspects of its use as an adjuvant have previously been documented [30,31]. For example, Spitzer et al. [30] reported that pluronic F127 in combination with a synthetic peptide from *Leishmania major* could elicit a Th1 response in mice and could elicit durable protection against this organism [30]. However, the effect of peptide alone was not included in this study so the exact role of F127 remains equivocal. Although in our studies addition of the immunomodulators chitosan and CpG enhanced the immunostimulatory capacity of F127 (Figures 4 and 5), F127 alone did also elicit a secondary response to rPA (see Figure 6b) and thus may play a role in the generation and/or recall of memory responses potentially by directing antigens and/or immunomodulators to immunologically relevant tissues.

Combinations of poloxamers, including pluronic F127, have recently been shown to enhance aspects of DNA delivery. For example, increased gene expression in mice of plasmid DNA *in vivo* occurred when the plasmid was formulated in combination of poloxamers that included F127 [32,33] and it was also reported that the mechanism of action centered on the ability to potentiate cellular uptake and to recruit and mature dendritic cells (DCs) [32]. However, these effects were optimal at very low, non-gelling concentrations (0.01% w/v) of poloxamers and thus similar mechanisms may not be operative in the current studies, in which we use much higher concentrations of F127 in combination with CpGs. Other work suggests that F127 can elicit hematopoiesis. For example, a recent study examined the bioavailability of and hematopoietic activity induced by Flt3 ligand (Flt3L) in mice. When delivered in an F127-based matrix, the F127 vehicle alone was found to cause a significant though modest increase in numbers of splenic colony forming units compared to control mice receiving BSS and this activity

could not be attributed to endotoxin contamination [34]. Data from a related study indicate that delivery of Flt3L in the F127-based matrix also enhanced numbers of mature DCs in the blood compared to Flt3L delivered in BSS.

However, in both these sets of studies, the formulations additionally contained hydroxypropylmethyl cellulose and therefore this activity cannot be definitively attributed to F127.

Significant enhancement was also seen in the antibody response to TT/F127/CpG versus TT/IFA/CpG over the first three months following single administration. IFA has been shown to cause a depot effect with antigen, thereby potentially allowing sustained release of antigen over an extended period of time. We also evaluated glycerol as an alternative delivery vehicle for TT/CpG because of its known protein stabilizing abilities [35,36] but this caused no enhancement of the anti-TT antibody response compared to TT/CpG alone. These data therefore suggest that the depot/stabilization effects are not sufficient to explain the enhancement obtained in the presence of F127. This strongly suggests that the F127 has some inherent properties allowing it to target the immune system. This is further supported by the work of Lemieux and co-workers [32] mentioned above and by our data showing that after a boost, anthrax rPA incorporated in the F127 matrix, without addition of other immunomodulators, elicited a substantial neutralizing antibody response (Figure 6b), which was equivalent to the secondary response elicited by rPA adsorbed to alum.

The currently available vaccine for anthrax (AVA or BioThrax^{TM}), which contains alum as an adjuvant, is considered safe and efficacious [37]. However, it has considerable drawbacks including poor standardization and the requirement for six immunizations

over an 18 month period followed by annual boosters to maintain an immune response commensurate with protection [38]. It has also been associated with a considerable number of side effects, ranging from mild local reactions to life-threatening reactions, such as anaphylaxis and shock [39]. Therefore, the Institute of Medicine has recommended that there is an urgent need for the development of a new vaccine.

Several second-generation vaccines based on purified rPA are currently under investigation and/or in clinical trials. Based on a number of animal models, including non-human primates, it is widely accepted that the humoral immune response, specifically anti-PA antibodies, plays a significant role in protection against anthrax. However, the level of anti-PA antibodies necessary to provide protective immunity and the role of cellular immunity are poorly defined. Based on these limitations it seems prudent to design a novel anthrax vaccine capable of inducing both a significant anti-PA antibody response and a cellular immune response. The F127/CpG formulation described here biased the immune response towards a Th1 response but not at the expense of the Th2 response as measured by IgG subclass analysis. Eight weeks after a single injection, the formulation containing rPA with F127/CpG induced toxin neutralizing titers that were significantly higher than all other formulations tested including the mix of rPA/CpG alone. Following a boost rPA/F127/CpG and rPA/CpG induced neutralizing antibody levels that were still significantly higher than levels induced by the other formulations tested although they were no longer significantly different from each other. The ability of the F127/CpG formulations to elicit neutralizing antibody responses and the ability of this formulation, as well as F127 alone, to generate immunological memory after a single

immunization, strongly suggests that F127 based formulations have potential for the generation of new and novel anthrax vaccine candidates.

Pluronic F127 belongs to a family of non-ionic block copolymers, known as poloxamers [3,40-46]. Other types of poloxamers have previously been used in various experimental vaccine formulations and have been shown to have potent adjuvant activity, e.g. CRL 1005 [47,48]. However, these polymers are very hydrophobic, having a much larger percentage of polyoxypropylene than F127, and they fail to exhibit reverse gelation characteristics. Furthermore it has been reported that the level of immunomodulatory activity of these polymers decreased when high percentages of POE were used [47]. In contrast, F127 acts as a sustained release vehicle and as a stabilizer for both antigen and adjuvant contained within the matrix. It is therefore distinct both chemically and functionally from these members of the poloxamer family that have previously been evaluated as vaccine delivery candidates.

In summary, our studies demonstrate the synergistic adjuvant effect of chitosan and CpGs co-administered with F127 after systemic administration of various protein antigens. In addition, F127 alone appears to play a role in establishing immunological memory. These promising results have encouraged us to investigate the use of this unique vaccine delivery system with a number of clinically relevant systemic and mucosal antigens, as well as with other adjuvants that could be potentially given at lower doses within the pluronic matrix.

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Table 1
ASC in the bone marrow and spleens of mice one year after immunization with TT/F127/chitosan or TT/PBS.

Source of Cells	*TT/F127/chitosan*			*TT/PBS*	
Bone marrow	952	384	404	56	120
Spleen	24	8	4	1	1

Bone marrow and spleens were obtained from Balb/c mice one year after a single s.c. immunization with 1.5 Lf TT in either F127/chitosan or PBS . ASC were enumerated by ELISPOT assay and data expressed as anti-TT specific ASC/10^6 cells for individual animals.

FIGURES

Figure 1

CD-1 mice (n = 8) were immunized once s.c. with 1.5 Lf TT/F127/chitosan (squares) or 1.5 Lf TT/AP (triangles). Serum samples were collected at various times and IgG anti-TT antibody levels measured by ELISA. Data are expressed as geometric mean titers of the IgG anti-TT antibody response on a log scale. Error bars represent standard deviations of the mean.

Figure 2

Balb/c mice were immunized either once s.c. with 1.5 Lf TT/F127/chitosan (n = 8) (squares) or three times (0, 4 and 8 weeks) with 1.5 Lf TT/AP (n = 4) (triangles) for a total of 4.5 Lf TT. Serum samples were collected at various times and IgG anti-TT antibody levels measured by ELISA. Data are expressed as geometric mean titers of the IgG anti-TT antibody response on a log scale. Error bars represent standard deviations of the mean.

Figure 3

Balb/c mice (n = 8) were immunized i.p. with 0.5 Lf TT in either PBS (diamonds) or F127/chitosan (squares) and were challenged at week 6 with 100 x LD_{50} tetanus toxin. Negative controls consisted of mice immunized i.p. with vehicle (F127/chitosan) only (open triangles). Survival was monitored for 8 days post challenge and deaths recorded.

Figure 4

Balb/c mice (n = 8) were immunized once s.c. with 0.5 Lf TT in either F127/chitosan, /chitosan or F127. Serum samples were collected at various times and IgG anti-TT antibody levels measured by ELISA.

Data are expressed as geometric mean titers of the IgG anti-TT antibody response. Error bars represent standard errors of the mean. Black bars: TT/F127/chitosan; white bars: TT/chitosan; gray bars: TT/F127.

Figure 5

Balb/c mice were immunized once s.c. with 0.5 Lf TT (A, C) or 1 Lf DT (B) in various formulations. Serum samples were collected and assayed for IgG antibodies by ELISA. *Panel A*: IgG anti-TT antibody responses from mice (n = 4) immunized with either TT/F127/CpG (diamonds), TT/IFA/CpG (triangles) or TT/CpG (squares). Data are expressed as geometric mean titers of the IgG anti-TT antibody responses on a log scale. Error bars represent standard deviations of the mean. *Panel B:* IgG anti-DT antibody responses from mice (n = 4) immunized 4 weeks previously. Open circles represent the titers of individual animals; bars represent the geometric mean titers for both groups. *Panel C:* IgG anti-TT antibody responses from mice (n = 8) immunized eight weeks previously either with TT/F127/CpG or TT/CpG at 2% (v/v) CpG or with TT/CpG (20% (v/v)) or with TT/F127 alone. Data are expressed as geometric mean titers of the IgG anti-TT antibody response. Error bars represent standard errors of the mean.

Figure 6

Balb/c mice (n = 6) were immunized with a single s.c. injection of 25 μg of rPA administered in F127, F127/chitosan, F127/CpG, CpG or alum and were boosted s.c. seven months later with the same formulation. Neutralizing antibody titers were measured by TNA in serum samples collected 8 weeks after primary immunization (A) and 2 weeks post boost (B). Open circles represent serum titers from individual mice normalized to a rabbit anti-rPA antiserum control; solid lines represent geometric means of individual normalized TNA values.

Figure 7

Levels of IgG subclasses were measured by ELISA in serum samples from mice immunized as described in Figures 5A (TT) and 6 (rPA). Data are expressed as geometric mean titers of the IgG anti-TT antibody responses on a log scale. Error bars represent standard deviations of the mean. Black bars: IgG1; white bars: IgG2a.